CELL GROWING AND DIFFERENTIATION MONITORING SYSTEM USING ELECTRICAL BIOIMPEDANCE SPECTROSCOPY MEASUREMENT ON INTERDIGITATED MICROELECTRODES.

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Abstract: The present work is focused on myocardium tissue regeneration using mesenchymal stem cells from bone marrow, which should differentiate to cardiac myocytes. In each part of the differentiation process of the cells, it is necessary the culture should be characterised, so off line and destructive techniques are commonly used. This work is aimed at improving these processes with the characterization of the structural properties obtained from non-destructive electrical measurements thus allowing continuous monitorization of the culture. Electrical impedance spectroscopy (EIS) will be used in the determination of passive electrical properties.

EIS measurements on monolayer animal cell cultures are usually performed using a two-wire strategy. Because of this, the measurement is very sensitive to the electrode covering ratio and to the degree of adherence of cells. Of course, these parameters give useful information but can mask the behaviour of the cell layer above the electrodes.

Several commercial microelectrode structures have been evaluated. The chosen configuration is a double interdigitated microelectrode set that allows the performance of both 2 and 4 wire measurements. The validation measurements have been performed with simulated grow processes using the settlement of cell suspensions and also with real cell growth (condrocyte and vero cells).

Introduction

Injured heart is not capable of self-regeneration. Cellular therapy based on the mobilization of autologous progenitor cells derived from bone marrow or based on exogenous administration of these precursors can be an efficient alternative to the current medical therapies. Cellular therapy may be performed applying isolated cells, as a patch in a therapeutic three-dimensional scaffold, as a patch using the recolonized wound or promoting changes in the scar which permits in vivo recolonization. The work we are presenting is a part of research project on myocardium regeneration using biocompatible scaffolds colonized by cardiac progenitors obtained from the differentiation of stem-cells. The project objectives are:

– To study processes such as the mobilization, attachment and functional implantation of endothelial progenitor cells in patients with or without dilated myocardiopathy.
– To induce the differentiation of mesenchymal stem cells from bone marrow to cardiac myocytes and characterize their electrophysiological and structural properties.
– To develop, through cooperation of endothelial and myocardiogenical progenitors, tissue construction of viable and functionally active cardiac myocytes in three-dimensional polymers and infarcted tissue scars: Cardiac tissue engineering.

The various techniques being used for the maintenance, manipulation and monitorization of cell cultures are characterized, from an instrumental point of view, for the following aspects:

– the need of the biological material characterization in each stage of the process.
– the use of techniques usually destructive to carry out this characterization.
– as any biological manipulation, they involve an intrinsic uncertainty in the results, so repeated experiments must be performed to obtain statistical parameters.

These facts show the need of a support system for the systematic performance of cell cultures. In this work, we present the initial stages of the use of macroscopic measurement techniques which permit on line monitoring of passive and active electrical properties of monolayer and three-dimensional cultures.

Electrical Impedance Spectroscopy (EIS) will be used to determine the passive electrical characteristics. Active properties will be characterised by acquiring the spontaneous and induced extracellular potentials using microelectrode arrays. In this work, we present the EIS measurement set-up and the preliminary results.

Electrical impedance measurements on monolayer animal cell cultures are usually performed with a two-wire strategy by using microelectrodes placed at the bottom of the culture plates [1], [2]. In this way, the

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resulting measured impedance is very sensitive to the microelectrode surface covered by cells and to the adherence of those cells. These features are of great interest by themselves [3] but introduce a source of indetermination if the goal is to obtain a quantitative measurement of the biomass growing in the layers above the microelectrode surface. An alternative is to use several electrode pairs, with varying interelectrode distance to achieve different penetration depth in the cell layers [4].

Several commercial microelectrode structures were evaluated and a double set of interdigitated microelectrodes (IME) was finally chosen. We obtained their electrical characteristics and evaluated the effect of their parameters on the 2 and 4 wire impedance measurement. With these results, a front-end stage was specified and designed to adapt the microelectrodes to an impedance analyzer.

Given that the use of real stem-cell experiments in the validation stage is too expensive and time consuming, we have first used the settlement of cell suspensions over the microelectrode surface as a quick model of the cell growing. After that phase, we have performed monolayer growing of condrocytes and vero cells.

Materials and Methods.

Microelectrodes

Given the myocyte size, we have chosen electrode structures with interelectrode distances in the 5 – 20 μm. Simple 4 independent electrode tracks and interdigitated structures have been studied. We have finally chosen the Interdigitated Microsensor Electrodes (IME) from Abtech [5]. There are 2, 3 and 4 Pt or ITO electrode structures deposed on a borosilicate glass (Schott) (Figure 1). The interelectrode distance and the track width is 15 μm. The length is 5 mm. According to the manufacturer information, they are intended to perform electrochemical measurements (including EIS) after placing electroactive layers on the IME surface.

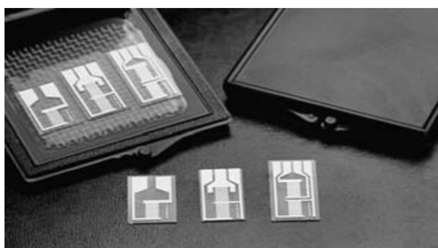

Figure 1: IME (2 ,3 and 4 electrodes) composed by interdigitated electrode pairs (Abtech)

Microelectrode Characterisation

The first stage of the electrode characterisation consists on the measurement of the electrode impedance and its dependence with medium conductivity, given the changes in this parameter in real conditions. Starting from the classical model (Figure 2) for the electrode impedance, the parallel resistance (R_{BF}) is neglected given that the electrodes are metallic (polarized) and the working frequency margin starts above 1 to 10 kHz.

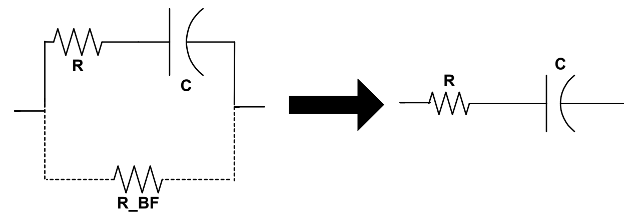

Figure 2: Microelectrode impedance electrical model

The measurement set-up prepared to characterise the microelectrodes included an impedance analyser which performed a frequency sweep up to 1 MHz. A front-end stage with switches allowed different electrode configuration measurements (2,3,4 wire). The capacitances between the different electrode pairs were also determined. Figure 3 shows the four impedance spectra corresponding to the electrode impedances of a double pair of interdigitated Pt microelectrodes. The corner frequency is placed around 300 Hz and the resistive value is quite small (≈ 50 Ω). The dispersion between them is not high. The capacitance between electrodes of the same pair is about 20 pF and lower than 10 pF if measured between electrodes of different pairs.

Several circuits have been simulated with the obtained parameters, including imbalances up to 50% for the electrode impedances in order to evaluate the front-end adequacy to the desired global system performance.

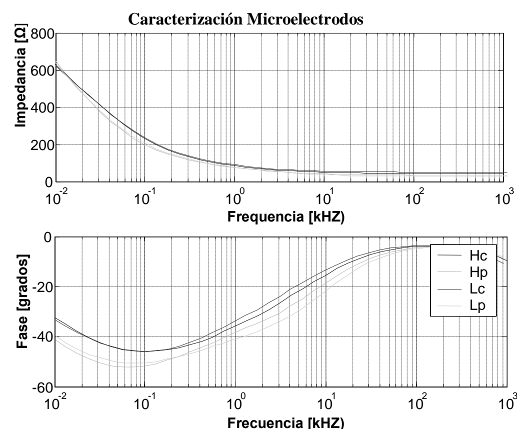

Figure 3: Electrode impedances of a double Pt IME pair

Front-End and Measurement System

The front-end includes a current injector, a transimpedance amplifier and a wide-bandwidth differential amplifier coupled in AC and with very high input impedance. The amplifier presents a CMRR of 95 dB al LF and 60 dB a 1 MHz without any adjustment. The front-end outputs are connected to an HP4192A impedance analyzer which is used as a vectorial voltmeter.

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Measurement Cells

To characterise monolayer growth, the microelectrodes are placed at the bottom of a Petri dish (3,5 cm ∅), (Figure 4). The contacts cross the wall through an aperture which is sealed with a biocompatible silicone compound (Sylgard 184, Corning Glass). Before starting the culture, and after inserting the IMEs in the bottom of the dishes, a cleaning protocol must be performed in order to avoid possible toxicities for the culture. This protocol consists in pouring into the dish 3-4 ml of distilled water and removing it after five minutes (repeating three times). Afterwards, adding 4 ml of distilled water and incubate over night. The day after, remove the water and clean with 3-4 ml of medium three times again. Afterwards, the dish with the IME is ready for culture.

The different dishes (measuring one and control dishes) must be inoculated from a common inoculum. Before connecting to the measuring system, 1-2 hours must be waited to let the cells attach to the surface in an defined horizontal position to allow homogeneous distribution among the IME surface. Then the impedance measurement is started. Experiments were carried out using both condrocyte and Vero cells.

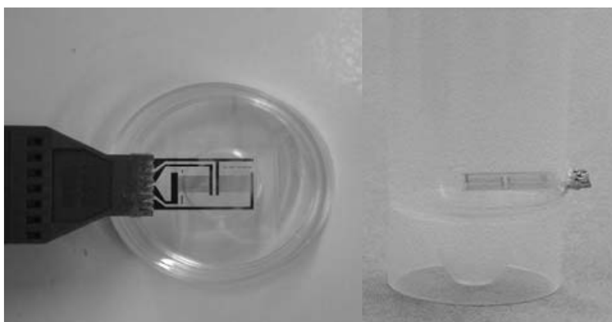

Figure 4: Electrodes on a Petri dish and in a Falcon tube

To perform the viability study of the described techniques using a quick and low-cost model, a second structure was implemented. To simulate the growing on the top layer of a microelectrode structure, we use the settlement process of a cell suspension during a time short enough (1-2 hour) respect to the duplication time of the cells. In addition, there is no need of using sterile conditions for this experiment. Both yeast cells and mesenchimal cells extracted from human blood have been used. The microelectrodes are placed in the bottom area of a Falcon tube (Figure 4).

Two cell suspensions were used: a yeast (Saccharomyces cerevisiae) suspension(40 g/l dw) in saline solution (2.5 g/l) for the preliminary experiments and system set-up and a suspension of mononuclear $CD34^-$ human cells extracted from blood that are similar to the mesenchimal cells used in the cell expansion and differentiation protocols of the research project. The suspensions are stirred and then left settling along 2 hours in the Falcon Tube at constant temperature. An impedance spectrum is obtained every 15 s between 1 kHz and 10 MHz at 8 points per decade.

Results

Figure 5 shows a condrocyte culture growing on the microelectrode surface. Several biocompatibility tests were performed before the measurement stage.

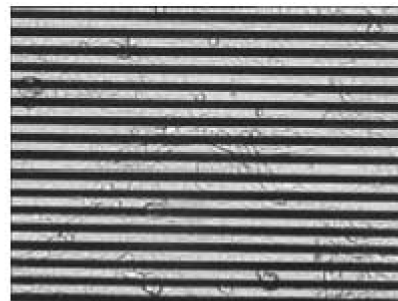

Figure5: Condrocytes growing on the IME surface.

Figure 6 and 7 show the two and four electrode measurements of CD34- mesenchymal cells settling on the electrodes. Two-wire measurements show a prevailing electrode impedance effect. In four-wire measurements, the relaxation due to the increment of cell density can be clearly observed although the electrode effect is still present. Figure 8 shows the biomass estimator E_2 [6] during a 10 hour measurement of a condrocyte culture in an incubator.

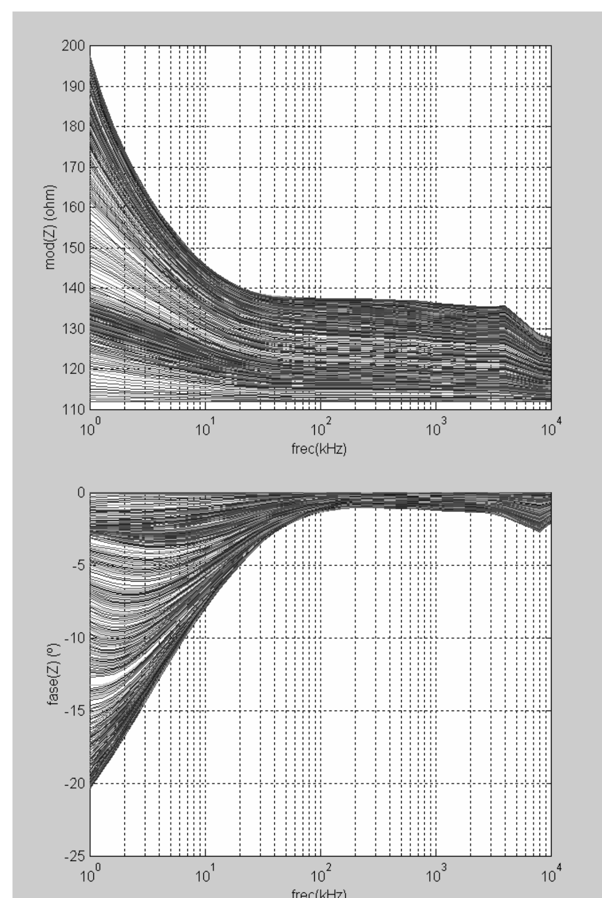

Figure6: Two-electrode EIS during mesenchymal cell suspension settling on the electrodes.

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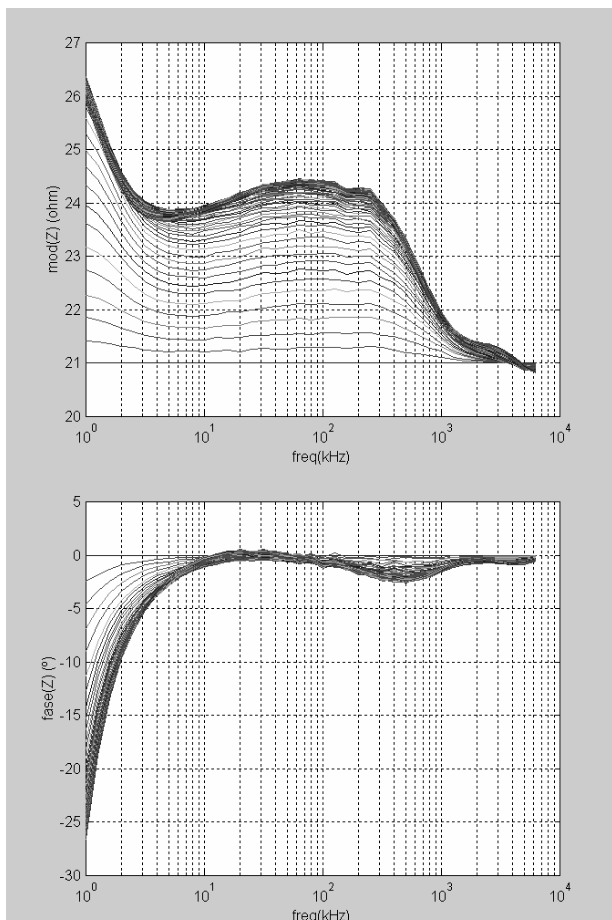

Figure7: Four-electrode EIS during the mesenchymal cell suspension settling on the electrodes.

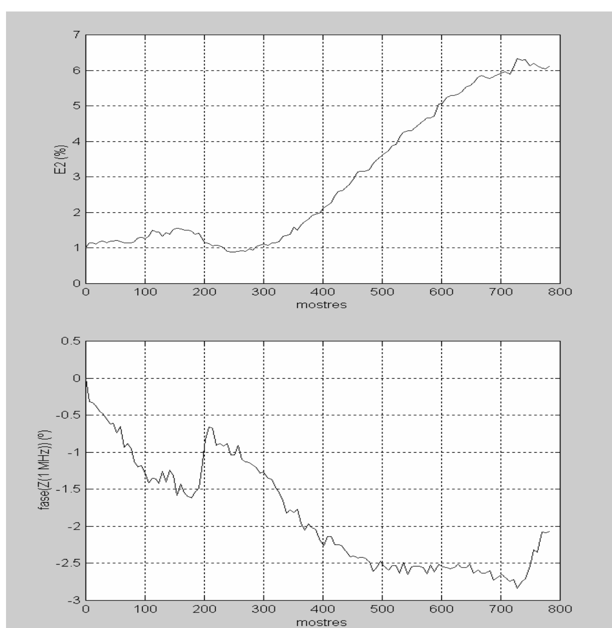

Figure8: Two-electrode EIS along settling of mesenchymal cell suspension on the electrodes.

Discussion and Conclusions

The double pairs of interdigitated microelectrodes, whose initial function was to perform differential measurements between two biosensors allow the realisation of both two and four electrode impedance measurement, giving characteristics of the electrode interface and the cell layer.

The two-wire measurements present a first stage in which the ratio of electrode area covered by cells is the dominant effect. In the simulated grow processes, this effect is monotonous but in real growths, the cell confluence can leave open spaces and present a time course not homogeneous. The impedances measured in this case are largely higher than those obtained with the 4-electrode technique, being the impedance electrode the dominant term and being its contribution to the overall impedance unknown and variable.

The combined use of two and four electrode measurements can provide structural information about the cell culture in a continuous and non-destructive way along the growth and differentiation processes in stem-cell experiments.

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